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Molecular Identification of Closely Related *Candida* Species Using Two Ribosomal Intergenic Spacer Fingerprinting Methods

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Recent changes in the epidemiology of candidiasis highlighted an increase in non-*Candida albicans* species emphasizing the need for reliable identification methods. Molecular diagnostics in fungal infections may improve species characterization, particularly in cases of the closely related species in the *Candida* complexes. We developed two PCR/restriction fragment length polymorphism assays, targeting either a part of the intergenic spacer 2 or the entire intergenic spacer (IGS) of ribosomal DNA using a panel of 270 isolates. A part of the intergenic spacer was used for discrimination between *C. albicans* and *C. dubliniensis* and between species of the *C. glabrata* complex (*C. glabrata/C. bracarensis/C. nivariensis).* The whole IGS was applied to *C. parapsilosis, C. metapsilosis,* and *C. orthopsilosis*, and to separate *C. famata* (*Debaryomyces hansenii*) from *C. guilliermondii* (*Pichia guilliermondii*) and from the other species within this complex (ie, *C. carpophila*, *C. fermentati* and *C. xestobii).* Sharing similar biochemical patterns, *Pichia norvegensis* and *C. inconspicua* exhibited specific IGS profiles. Our study confirmed that isolates of *C. guilliermondii* were frequently misidentified as *C. famata*. As much as 67% of the clinical isolates phenotypically determined as *C. famata* were recognized mostly as true *P. guilliermondii*. Conversely, 44% of the isolates initially identified as *C. guilliermondii* were corrected by the IGS fingerprints as *C. parapsilosis*, *C. fermentati*, or *C. zeylanoides*. These two PCR/restriction fragment length polymorphism methods may be used as reference tools [either alternatively or adjunctively to the existing ribosomal DNA (26S or ITS) sequence comparisons] for unambiguous determination of the *Candida* species for which phenotypic characterization remains problematic. ***(J Mol Diagn 2011, 13:12–22; DOI: 10.1016/j.jmoldx.2010.11.014)***

Current changes in the epidemiology of invasive mycoses highlighted a shift in the *Candida* species involved with a reduced proportion of *C. albicans* and an increase in non-*C. albicans* species.[1–4] In the most recent series, including the large cohort of 2019 patients with candidemia enrolled from 2004 through 2008, *C. albicans* accounts for less than one half of the isolates.[3,5–12] Although *C. albicans* antifungal susceptibility remains the rule, and reports on resistant isolates are very scarce, other species such as *C. krusei*, *C. glabrata, C. bracarensis, C. nivariensis, C. parapsilosis*, and *C. guilliermondii* are either innately resistant or show decreased susceptibility patterns to azoles, amphotericin B, or echinocandins.[13–21] Thus, the therapeutic impact of this shift might be critical and should be considered in patient management. Consistent with this trend, the recent revision of the consensus guidelines actually recommends an adjustment of the treatment according to the isolated *Candida* species.[22] In yeasts, there is no transfer of resistance between cells and acquisition of resistance, which is mainly observed in restricted clinical settings such as allogeneic blood marrow transplant or AIDS patients under sustained prolonged azole treatment.[5,14,23] Therefore, species identification remains basically predictive of drug susceptibility. Current methods for yeast identification in clinical practice are based on phenotypic features and carbohydrate assimilation tests that require 2 to 5 days or even longer in the case of unusual species.[24,25] These phenotypic methods including the automated ones may lead to mis-identification, particularly in the case of the closely related species.[16,18,26–31] Several molecular approaches have been developed and were designed mostly for the ribosomal RNA (rRNA) genes: targeting either the D1D2 domain of the 26S rRNA large subunit or the internal transcribed spacer regions ITS1

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and ITS2.[25,29,30,32,33] Restriction enzyme analysis of the intergenic spacer 1 (IGS1) region from the 26S to the 5S ribosomal DNA (rDNA), was used for identifying species of the *Saccharomyces sensu stricto* complex.[34] Later, the NTS2 region (from 5S to ETS1) part of the IGS2 was shown to be more appropriate.[35] Several basidiomycetous yeasts include the pathogen *Cryptococcus neoformans* and *Trichosporon* species may also be determined by using the IGS1 or the whole IGS region from the 26S to the 18S rRNA genes.[36–38] In the same way, it has been reported that IGS fingerprints are reliable to distinguish *Candida famata* var. *famata* from *Candida famata* var. *flareri* and 21 other species of the genus *Debaryomyces*.[39] Thus, PCR/restriction fragment length polymorphism (RFLP) fingerprints or sequencing of the IGS domain[39] can be used as alternative or adjunct to D1D2 sequence (26S rDNA)[40,41] or ITS sequencing.[30,33,42]

Here, we selected primers for partial amplification of the IGS (IGS2) and we established the specific patterns of *C. albicans, C. dubliniensis*, *C. glabrata, C. bracarensis, C. nivariensis* and *C. tropicalis*. Other primers, described in reference 39, were used to amplify the complete IGS domain leading to the discrimination of other closely related yeast species: *C. parapsilosis, C. metapsilosis, C. orthopsilosis, C. famata (Debaryomyces hansenii), C. guilliermondii (Pichia guilliermondii*), *C. carpophila*, *C. fermentati*, *C. xestobii*, *P. norvegensis*, *C. inconspicua, Clavispora lusitaniae, C. pararugosa, C. rugosa, C. catenulata, C. zeylanoides, Kluyveromyces marxianus, K. lactis, C. palmioleophila, C. pseudoglaebosa*, and *Saccharomyces cerevisiae*. We further report on the evaluation of this new set of two PCR/RFLP methods for accurate identification of the *Candida* species for which phenotypic characterization remains uncertain.

Materials and Methods

Yeast Strains and Isolates

A panel of 270 isolates of most medically relevant species was investigated. Isolates are listed in Tables 1 and 2, as well as Supplemental Tables S1 and S2 (*http://jmd.amjpathol.org*). Reference strains were from The Centraalbureau voor Schimmelcultures (CBS) collection and the Belgian Co-ordinated Collections of Micro-organisms/IPH-Mycology (BCCM/IHEM) public collection and were kindly provided by Dr. Hiroshi Fukuhara and Dr. Françoise Symoens, respectively, or were purchased directly. Clinical isolates were collected mostly in mycology laboratories of Hôtel-Dieu in Paris and Lille University Hospital, France.

Preliminary Phenotypic Identification of Clinical Isolates

Clinical isolates were routinely cultured either on Sabouraud dextrose agar supplemented with chloramphenicol and gentamicin (Bio-Rad; Marnes-La-Coquette, France) or on the chromogenic medium BBL CHROMagar Candida plates (BD Biosciences, Le Pont de Claix, France) for presumptive identification of *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. glabrata*. Identification was further confirmed by Auxacolor (Bio-Rad) or ID32C (bioMérieux, Marcy-l'Étoile, France) systems, based on assimilation of carbohydrates. The Glabrata R.T.T. test, based on the trehalase detection (Fumouze, Levallois-Perret, France) or the latex agglutination-based test Bichro-Dubli (Fumouze) were used for confirmation of *C. glabrata* and *C. dubliniensis*, respectively. Other yeast species were identified by phenotypic tests with the Auxacolor or the ID32C (bioMérieux) systems.

Fungal DNA Extraction

For clinical isolates, direct PCR amplification was performed with a single colony re-suspended in 20 μl of sterile water, supplemented with RNase (1 μl of RNase solution 10 mg/ml) (Roche, Meylan, France), heated for 5 minutes at 95°C, then 5 μl were added to the PCR mixture as previously described.[33] For the reference strains and some clinical isolates for which the preceeding technique failed to give positive PCR, genomic DNA was extracted as previously described.[39]

Primer Selection and PCR Amplification

The primers designed for the IGS2 method were NTUni 5′-TTAACTACAGTTGATCGGAC-3′-selected from the 5S conserved sequence of *S. cerevisiae/D. fabryi* (nucleotides 65-85)[39] and CA18R0 5′-GCAGTTTCACTGTATA-AATTG-3′ from the 18S rRNA sequence of *C. albicans* (nuclotides 58-78). The primer pair for the whole IGS was LR13-SR21 as previously described.[39] PCR amplification was performed in an AB 2400 Thermo Cycler (Applied Biosystems, Les Ulis, France). Conditions applied for both PCR methods were as follows: initial denaturation at 94°C for 4 minutes, followed by 30 cycles of 30 seconds at 94°C; 30 seconds at 48°C; 1 minute at 72°C, and a final elongation step of 5 minutes at 72°C. The mixture contained 5 pmoles of each primer, 5 nmoles of dNTP, 1.5 U Ex*Taq* polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan) in its 1X buffer, 50 ng of DNA from yeast reference strains, or 5 μl of the thermolysed colony suspension for clinical isolates, in a final volume of 50 μl.

After amplification, 2 μl of the PCR product was checked in 0.7% agarose gel and 5 μl of the amplification product was used without purification for restriction with *Nla*III (Bio-Labs, Hitchin, UK) or *Alu*I (Invitrogen; Cergy Pontoise, France) for the IGS2 and the IGS products, respectively, using 1.5 units for 90 minutes at 37°C for both digestion reaction. The subfragments were separated on 1.15% agarose gel containing ethidium bromide in Tris-Borate-EDTA 0.5 X. After 2 hours running at 120 V, the gel was stained in an ethidium bromide bath, de-stained in water, and observed under UV light. For IGS2 fingerprinting, we choose NlaIII restriction enzyme because the IGS2 sequences presented more NlaIII sites (CATG) than *Alu*I ones (AGCT).

The time to generate an entirely in-house result, from isolation to definitive identification was estimated to be a minimum of 7 hours (10 strains per run) and the cost is estimated at appoximately $4.00 for each identification from the culture to the profile compared with $13.20 when two reads are needed to obtain the D1D2 sequence.

Table 1. List of Type and Collection Strains of *Candida spp.* Identified by the PCR/RFLP Method

Species/Strain*	PCR/RFLP profile accession number†	PCR/RFLP identification (this study)
C. albicans		
CBS 562§, CBS 1893, CBS 5982, CBS 5983, CBS 6431, CBS 8190	ALBI FN554375	*C. albicans*
SC5314, CBS 1949, CBS 2697, CBS 2707	ALBI‡ FN554376	*C. albicans*
C. dubliniensis		
CBS 7987§, CBS 7988, CBS 8500¶, CBS 8501¶	DUBL FN554377	*C. dubliniensis*
C. glabrata		
CBS 138§, CBS 860, CBS 861, CBS 1528, CBS 2175, CBS 4692, CBS 7904, CBS 5691	GLAB FN554379	*C. glabrata*
C. bracarensis		
CBS 10154§	BRAC FN554380	*C. bracarensis*
C. nivariensis		
CBS 9983§, CBS 9984¶, CBS 9985¶	NIVA FN554381	*C. nivariensis*
C. parapsilosis		
CBS 604§, CBS 1954, CBS 2152, CBS 2193, CBS 2194, CBS 2195, CBS 2197, CBS 2211, CBS 2215, CBS 2916, CBS 5301, CBS 6318, CBS 8050	PPSI	*C. parapsilosis*
C. orthopsilosis		
NCPF 8795	MPSI	*C. orthopsilosis*
C. metapsilosis		
NCPF 8789	OPSI	*C. metapsilosis*
Debaryomyces hansenii		
CBS 767§, IHEM 711¶, IHEM 3438¶, IHEM 5768¶, IHEM 6275¶, IHEM 6826¶, IHEM 10430¶‖	DEHA	*D. hansenii var. hansenii***
Debaryomyces hansenii lineage *Candida famata*		
CBS 1795§	CAFA AM992926	*C. famata var. famata***
Pichia guilliermondii		
CBS 2030§, CBS 2021, CBS 2024, CBS 2077, CBS 2083, CBS 2084, CBS 2672¶, CBS 4236, CBS 5265, CBS 5674, CBS 6109¶††, CBS 6557, CBS 7099, CBS 7232	PIGU AM992960	*P. guilliermondii*
Candida guilliermondii		
CBS 566§	PIGU	*C. guilliermondii*
C. carpophila		
CBS 5256§, CBS 5258¶, CBS 7921¶, CBS 5257¶	CARP FN554237	*C. carpophila*
C. fermentati (= *Pichia caribbica*)		
CBS 2022§, CBS 5059¶, CBS 5241¶‡‡, CBS 6319¶, CBS 8302¶, CBS 8303¶	FERM FN554235	*C. fermentati*
C. xestobii		
CBS 5975§	XEST FN554238	*C. xestobii*
Pichia norvegensis		
CBS 6564§, CBS 1911, CBS 1953, CBS 2327, CBS 5304, CBS 6917	PINO FN554245	*P. norvegensis*
CBS 1921, CBS 2125, CBS 2145, CBS 6639	PINO(‡) FN554246	*P. norvegensis*
CBS 2126	PINO(‡)	*P. norvegensis*
CBS 2128, CBS 2144	PINO(‡)	*P. norvegensis*
Candida norvegensis		
CBS 1922§¶, DBVPG 6871§¶	PINO	*P. norvegensis*
CBS 1922§§	ZEYL FN554768	*C. zeylanoides*
C. inconspicua		
CBS 180§, CBS 990, CBS 2833	INCO FN554239	*C. inconspicua*
CBS 620	INCO(‡)	*C. inconspicua*

*For collections and origin of the strains, see websites, last accession: July 16, 2010; CBS (*www.cbs.knaw.nl/yeast/BioloMICS.aspx*; *http://www.cbs.knaw.nl/yeast/BioloMICS.aspx*), DBVPG, (*www.agr.unipg.it/dbvpg/*), and BCCM/IHEM, (*http://bccm.belspo.be/about/ihem.php#researc*), NCPF, (*http://www.hpacultures.org.uk/*).
†Sequences with accession number starting with FN were determined in this study and are deposited in Gen Bank.
‡Polymorphic variant of the type strain pattern.
§Type strain.
¶Strains received from CBS, DBVPG, or IHEM.
‖Used as reference strain of *C. famata*.
**See Ref 38 for new nomenclature
††As *C. carpophila* by the CBS
‡‡As *P. guilliermondii* by the CBS
§§Strain from Dr. Hiroshi Fukuhara

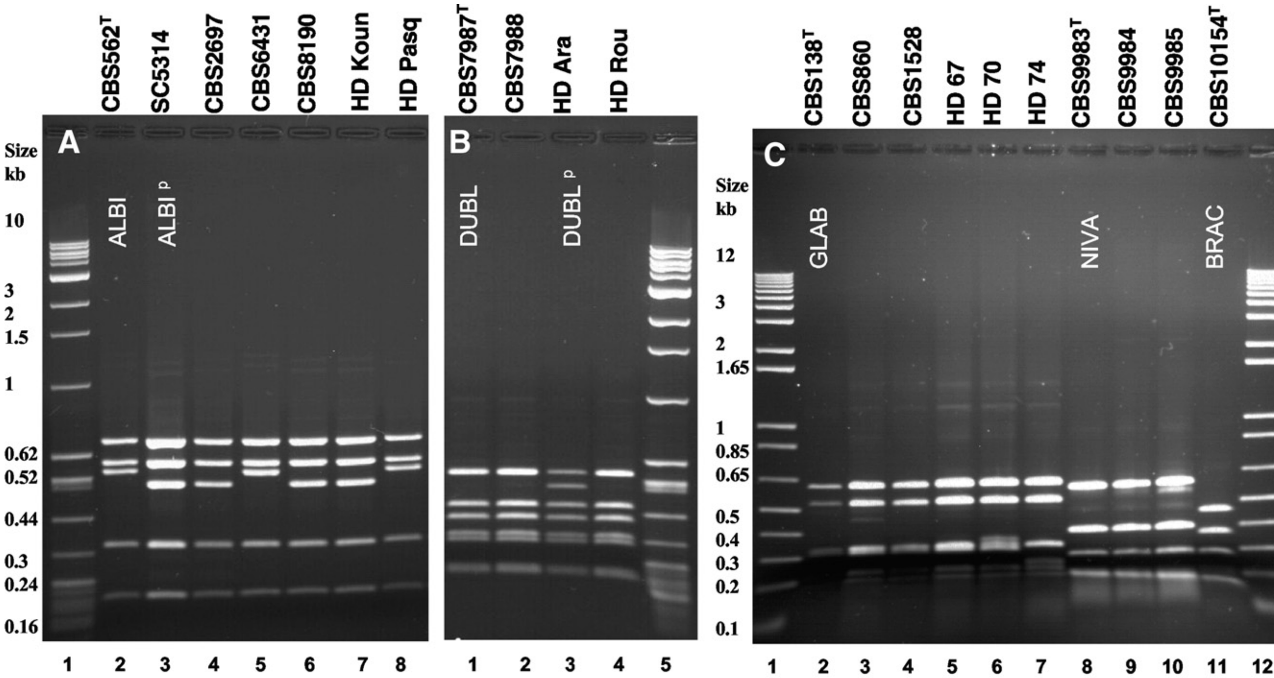
Table 2. List of Clinical and Environmental *Candida* Isolates

First identification/Strain	Site of isolation	PCR/RFLP profile accession number	PCR/RFLP (this study) identification
Candida albicans			
HD Pasq	Mouth	ALBI	*C. albicans*
HD Koun	Mouth	ALBI*	*C. albicans*
HD 10	Biliary fluid	ALBI	*C. albicans*
HD 13, HD 31, HD 38	BAL†	ALBI	*C. albicans*
HD 14	BA‡	ALBI	*C. albicans*
HD 37	Sputum	ALBI*	*C. albicans*
C. dubliniensis			
HD Rou	Mouth	DUBL	*C. dubliniensis*
HD Ara, Lil 7	Mouth	DUBL* FN554378	*C. dubliniensis*
L 1, L 3, L 10, L 480, L 513	Sputum	DUBL	*C. dubliniensis*
L 11	Trachea	DUBL*	*C. dubliniensis*
L 12	Stool	DUBL	*C. dubliniensis*
L 16	Vagina	DUBL	*C. dubliniensis*
L 479, L 481, L 512	Tongue	DUBL	*C. dubliniensis*
L 522	Tongue	DUBL*	*C. dubliniensis*
C. glabrata			
HD 67, HD 68, HD 69, HD 71, HD 73	BA	GLAB	*C. glabrata*
HD 70, HD 72	Sputum	GLAB	*C. glabrata*
HD 74, HD 75	Stool	GLAB	*C. glabrata*
C. parapsilosis			
MC 2, MC 7, MC 18	BA	PPSI	*C. parapsilosis*
M 3, MC 8, MC 58	Blood	PPSI	*C. parapsilosis*
AM06/0207			
C. inconspicua		INCO	*C. inconspicua*
MC 1	Blood	FN554240	
8121 4335			
Debaryomyces hansenii/Candida famata			
Cfa4	Sputum	DEHA	*D. hansenii var. hansenii*§
Cfa 6, Boc1128	Mouth	CAFA	*C. famata var. famata*§
Cfa 2, CHR7305500	Skin	PIGU	*Pichia guilliermondii*
Cfa 5	Nail	PIGU	*P. guilliermondii*
CHR6009704	Sputum	PGLA	*C. pseudoglaebosa*
CHR6005538	Skin	PALM	*C. palmiolephila*
Cfa 3	Stool	KLMA	*K. marxianus*
Pichia guilliermondii			
HD 354	Sputum	PIGU	*P. guilliermondii*
HD 520	BAL	PIGU	*P. guilliermondii*
CHR004648, CHR993206, CHR00555181, CHR06173	Blood	PIGU FN554234	*P. guilliermondii*
CHR5000373, CHR7009193, CHR7005511	Trachea	PIGU	*P. guilliermondii*
CHR6006940	Tongue	PIGU	*P. guilliermondii*
CHR6008052	Gastric fluid	PIGU	*P. guilliermondii*
CHR6008164, CHR700196, CHR3009257	Mouth	PIGU	*P. guilliermondii*
CHR4006637	Bronchial aspirate	FERM	*C. fermentati*
CHR3008385	Stool	FERM FN554236	*C. fermentati*
Wild strains			
CXB5	Green Lemon skin, Viet Nam	PIGU	*P. guilliermondii*
CXB2, CXB7	Green Lemon skin, Viet Nam	FERM	*C. fermentati*
XT1	Mango skin, Viet Nam	PPSI FN554242	*C. parapsilosis*
CXB6, CXB13	Green Lemon skin, Viet Nam	ZEYL	*C. zeylanoides*
Kam494, Kam522, Kam531, Kam544, Kam592	Wild, Kamchatska, Russia	PPSI	*C. parapsilosis*

*Polymorphic variant of the type strain pattern.
†Bronchoalveolar lavage.
‡Bronchial aspirate.
§See Ref. 39 for new nomenclature.

Fingerprints and Band Pattern Analysis

The gel patterns were analyzed first by eye through comparison with the fingerprints of the reference strains or type strains run on the same gel. The strains chosen for comparison were selected according to presumptive identification and based on the size of the amplicons obtained by both IGS and IGS2 PCR. In addition, the gel patterns were digitally recorded and normalized for bp measurements using the standards included in each gel and the Vilber Lourmat BioGene 11.3 software (Vilber Lourmat, Marne-la-Vallée, France). Then the profiles of the target



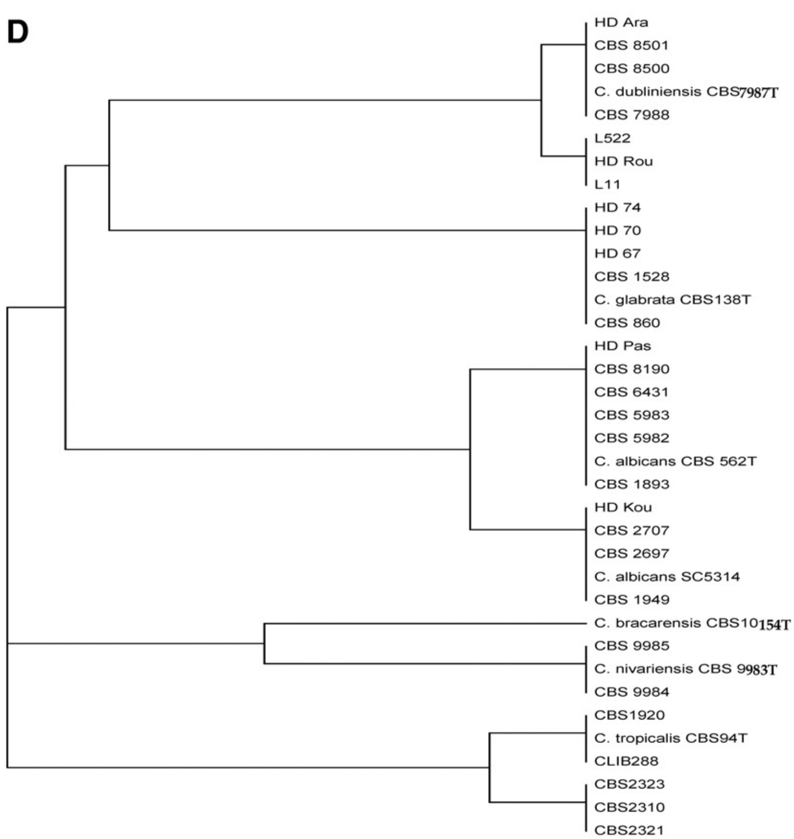
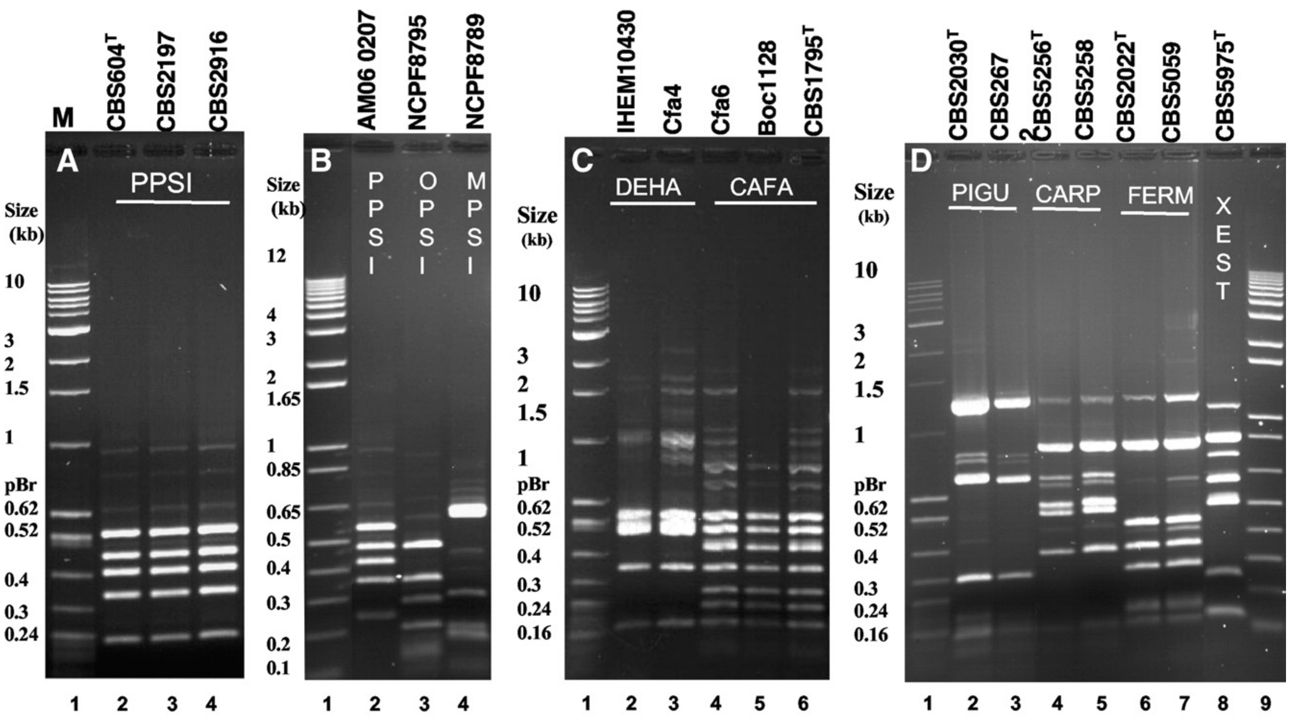


Figure 1. Comparison of *Nla*III fingerprints of IGS2 amplicons from type strains, collection strains, and clinical isolates. **A:** *Candida albicans* profiles. Lane 1, size marker (1 kb ladder and pBr322-MspI); ALBI profile shown by the *C. albicans* CBS 562^T type strain (lane 2), CBS 6431 (lane 5), and the *C. albicans* clinical isolate HD Pasq (lane 8). ALBI^P profile illustrated by the *C. albicans* SC5314 reference strain (lane 3), strains CBS 2697 (lane 4), CBS 8190 (lane 6), and the clinical isolate HD Koun (lane 7). **B:** The *C. dubliniensis* (DUBL) profiles. The DUBL profile shown by the *C. dubliniensis* CBS 7987^T type strain (lane 1), strain CBS 7988 (lane 2), and the *C. dubliniensis* HD Rou clinical isolate (lane 4). The DUBL^P profile illustrated by the *C. dubliniensis* HD Ara clinical isolate (lane 3). Lane 5, size marker (1 kb ladder and pBr322-MspI); **C:** Profiles of the species among the *C. glabrata* (GLAB)complex. Lane 1, 1 kb + size marker: Invitrogen 1 kb + λ ladder; the GLAB profile illustrated by the *C. glabrata* CBS 138^T type strain (lane 2), strains CBS 860, CBS 1528, the *C. glabrata* HD67, HD70, and HD74 clinical isolates (lanes 3 to 7); the *C. nivariensis* (NIVA) profile shown by *C. nivariensis* CBS 9983^T type strain (lane 8) strains CBS 9984 and CBS 9985 (lanes 9 and 10, respectively), and the BRAC profile illustrated by the *C. bracarensis* CBS 10154^T type strain (lane 11). Lane 12, 1 kb + size marker: invitrogen 1 kb + λ ladder. **D:** The IGS2/RFLP-based dendrogram drawn with the unweighted pair group method with arithmetic mean method. Distances were calculated using the Nei and Li/Dice method. Lower bar indicates relative genetic distance.

strains were recorded in a composite file using a binary code (1/0) containing the data of all of the reference/type strains. Genetic distances were calculated with the FreeTree software, which constructed a distance/similarity matrix (Nei and Li/Dice method).[43] The RFLP-based dendrograms using the unweighted pair group method with arithmetic mean method were drawn using the TreeView software.[44,45]



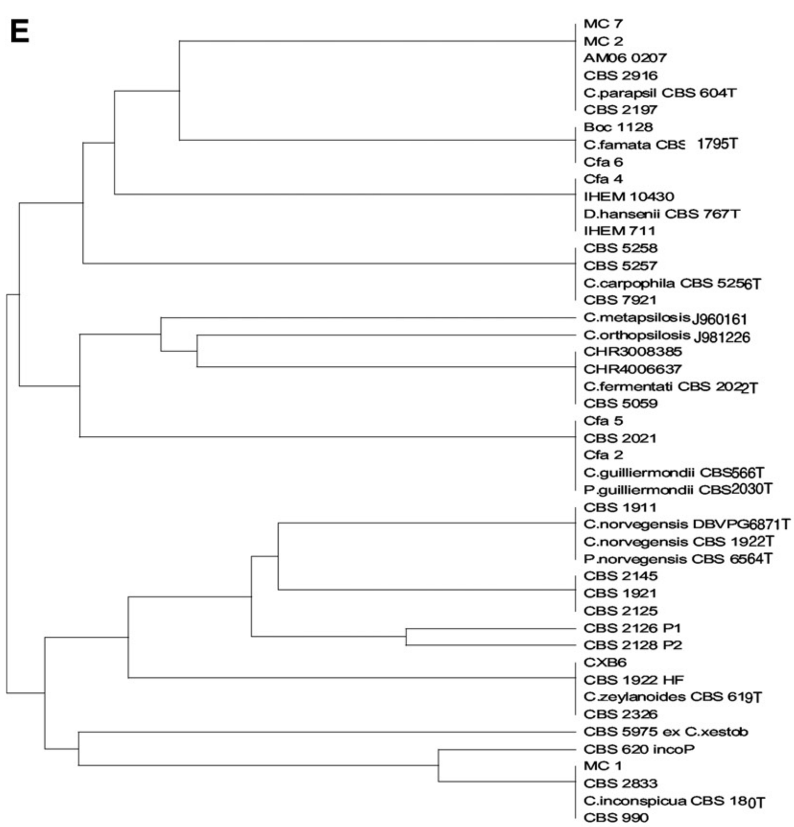


Figure 2. *Alu*I fingerprints of IGS amplicons from type strains, collection strains, and clinical isolates. **A:** Profiles of the species among the *C. parapsilosis* complex. Lane 1 1 kb + size marker: invitrogen 1 kb+ λ ladder, *C. parapsilosis* CBS 604^{T} type strain (lane 2) strains CBS 2197 and CBS 2916 (lanes 3 and 4). **B:** Lane 1, 1 kb + size marker: invitrogen 1 kb + λ ladder *C. orthopsilosis* strain NCPF 8795 (lane 3), and *C. metapsilosis* strain NCPF 8789 (lane 4). **C:** *Debaryomyces hansenii* and *C. famata* profiles. Lane 1, size marker (1 kb ladder and pBr322-MspI). The *D. hansenii* profile shown by the *D. hansenii* IHEM 10430 reference strain (lane 2) and the Cfa4 clinical isolate (lane 3). The *C. famata* profile shown by the *C. famata* CBS 1795^{T} type strain (lane 6), the Cfa6, and Boc1128 clinical isolates (lanes 4 and 5, respectively). **D:** Profiles of the species among the *Pichia guilliermondii* complex. Lane 1, size marker (1 kb ladder and pBr322-MspI). The *P. guilliermondii* profile is shown by the *P. guilliermondii* CBS 2030^{T} type strain (lane 2), and the *P. guilliermondii* strains CBS 2672 and CBS (lanes 3 and 4). The *C. carpophila* (CARP) profile illustrated by the *C. carpophila* CBS 5256^{T} type strain (lane 5) and strain CBS 5258 (lane 6). The *C. fermentati* (FERM) profile illustrated by the *C. fermentati* CBS 2022^{T} type strain (lane 6), the *C. fermentati* strains CBS 5059 (lane7), reference strains. The *C. xestobii* (XEST) profile shown by the *C. xestobii*-type strain CBS 5975^{T} (lane 8). **E:** The IGSAF/RFLP-based dendrogram drawn with the unweighted pair group method with arithmetic mean method. Distances were calculated using the Nei and Li/Dice method. Lower bar indicates relative genetic distance.

Sequencing and Sequence Analyses

The PCR products were used for sequencing (Cogenics, Meylan, France). The D1D2 domains were amplified and sequenced using the primer pair NL1/NL4.[46] Sequences were analyzed with the Staden package[47] and the GCG Wisconsin package (Madison, WI). The IGS sequences were deposited in the EMBL database sequence and accession numbers are listed in Tables 1 and 2, except for the strain CBS 1922^{HF} for which the D1D2 sequence was deposited (accession number FN554768).

Results

A total of 158 collection type strains, and 101 clinical and 11 wild isolates were fingerprinted by PCR/RFLP.

Using the IGS2 specific primers (5S-18S), the five pathogenic *Candida* species that compose the *C. albicans* and the *C. glabrata* complexes could be amplified. Examples of RFLP patterns of reference strains and of clinical isolates after *Nla*III restriction are shown in Figure 1A for *C. albicans,* Figure 1B for *C. dubliniensis*, and in Figure 1C for *C. glabrata*, *C. bracarensis,* and *C. nivariensis*. Figure 1D shows the IGS2-RFLP-based dendrogram drawn with these examples. Two different profiles were observed for *C. albicans* strains: ALBI and ALBI$^{(P)}$, represented by CBS 562^{T} and SC5314, respectively (Figures 1A and 1D). These patterns could easily be differentiated from those of *C. dubliniensis* for which two other distinct profiles were observed: DUBL as CBS 7987^{T} and DUBL$^{(P)}$ as the clinical isolate HD Ara (Figure 1B). The DUBL$^{(P)}$ pattern was restricted to clinical isolates (Table 2 and Figure 1D). Among the *C. glabrata* complex, all *C. glabrata* strains showed the same GLAB profile as the type strain CBS 138^{T} (Figure 1C and Tables 1 and 2). Notably, C*. bracarensis* and *C. nivariensis* type strains and collection strains showed two specific profiles, BRAC and NIVA, respectively, that were both different from the one of *C. glabrata* (Figures 1C and 1D).

As adjuncts to IGS2 patterns, fingerprints of the whole IGS were designed to separate the closely related species among the *C. parapsilosis* complex (Figure 2A). All *C. parapsilosis* strains, including the type strain CBS 604^{T} (Tables 1 and 2) showed the same profile PPSI. This profile was clearly divergent from those of OPSI and MPSI obtained with *C. orthopsilosis* and *C. metapsilosis,* respectively (Figure 2B). This IGS method could also easily differentiate *Debaryomyces hansenii (C. famata*) from *Pichia guilliermondii (C. guilliermondii*) (see the *D. hansenii, C. famata,* and *P. guilliermondii* profiles, respectively, in Figures 2C and 2D). The IGS fingerprints obtained with species belonging to the *C. guilliermondii* complex (ie, *P. guilliermondii*, *C. carpophila, C. fermentati*, and *C. xestobii*) were also distinct (Figure 2D). As the IGS amplification and *Alu*I fingerprinting (IGSAF) method was previously shown to differentiate *C. famata* from *D. hansenii*,[39] we used it to re-identify *C. famata* clinical isolates in parallel to reference strains fingerprints. Figure 2E shows the IGSAF-RFLP-based dendrogram drawn with representative strains identified by the IGSAF method.

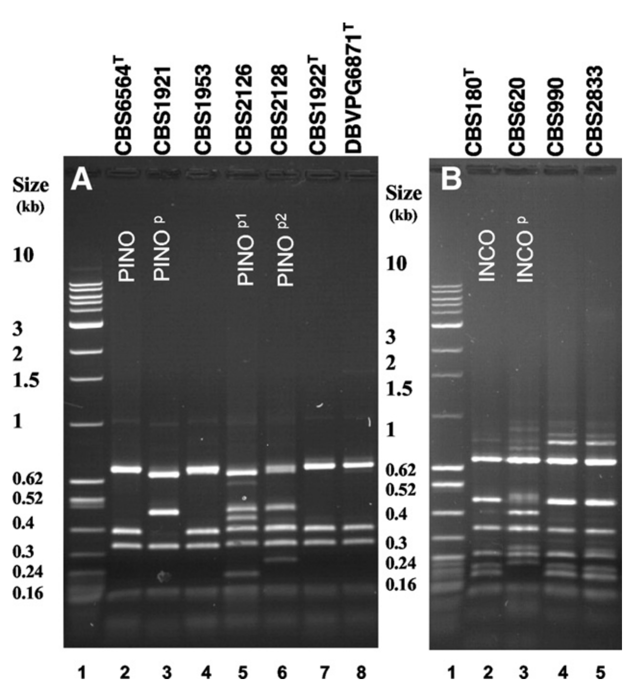


Figure 3. *Alu*I fingerprints of IGS products of type strains, collection strains and clinical isolates. **A:** The *P. norvegensis* profiles. Lane 1, size marker (1 kb ladder and pBr322-MspI). The *P. norvegensis* (PINO) profile shown by the *P. norvegensis* CBS 6564^{T}, *C. norvegensis* CBS 1922 ^{T}, DBVPG 6371^{T} type strains and the CBS 1953 (lanes 2, 7, 8, and 4 respectively), the PINO^{P} profile shown by the CBS 1921 (lane 3), the PINO^{P1} profile illustrated by CBS 2126 (lane 5), and the PINO^{P2} profile illustrated by CBS 2128 (lane 6). **B:** The *C. inconspicua* profiles. Lane 1, size marker (1 kb ladder and pBr322-MspI). The *C. inconspicua* (INCO) profile shown by the *C. inconspicua* CBS 180 ^{T} type strain (lane 2), strains CBS 990 and CBS 2833 (lanes 4 and 5). The INCO^{P} profile is shown as the CBS 620 (lane 3) reference strains.

Among the nine clinical isolates phenotypically identified as *C. famata* (Table 2), only two exhibited the IGSAF profiles similar to *C. famata* var. *famata* type strain CBS 1795^{T} (compare on Figure 2C, Cfa6 and Boc1128 to CBS 1795^{T}) and one as *D. hansenii* var*. hansenii* (compare on Figure 2B, Cfa4, and IHEM 10430). Among the others, three exhibited the *P. guilliermondii* pattern as *P. guilliermondii* type strain (Figure 2D, lane 2), one exhibited the *C. pseudoglaebosa* profile, and one exhibited the *C. palmioleophila,* and another strain exhibited a pattern similar to *K. marxianus* (*C. kefyr*) later confirmed by D1D2 sequencing (data not shown) (Table 2, Figure 2E).

Thus, the IGSAF method corrected *D. hansenii/C. famata* mis-identifications that affected 67% of the clinical isolates. All reference strains of *P. guilliermondii* and its anamorph *C. guilliermondii* showed the same *P. guilliermondii* fingerprint (Table 1, Figure 2E). As much as 44% (12/27) of the clinical and environmental isolates phenotypically identified as *C. guilliermondii* were recognized as *C. parapsilosis* (50%), as *C. fermentati* (33%), or as *C. zeylanoides* (17%) by the IGS method (Table 2 and Figure S1, *see http://jmd.amjpathol.org* for *C*. *zeylanoides* fingerprints). For *P. norvegensis* and *C. norvegensis,* no clinical isolates were available, and the collection strains revealed at least four profiles (Table 1, Figure 3A and Figure 2E): *P. norvegensis* profile (PINO) (eg, CBS 6564^{T}

Table 3. Amplicon Size According to the *Candida* Species Obtained with the Two PCR Methods

	PCR IGS2 product length		PCR IGS product length	
Species	Gel in kb*	Sequence in bp†	Gel in kb*	Sequence in bp†
Candida albicans	2.4	2.328		
C. dubliniensis	2.4	2.357		
C. bracarensis	2.3	2.191		
C. nivariensis	2.3	2.134		
C. glabrata	2.4	2.314		
C. tropicalis	2.4	2.242		
C. parapsilosis	1.4		2.1	2.084
C. metapsilosis	1.6		2.1	2.165
C. orthopsilosis	1.35		1.5	1.565
Debaryomyces hansenii	2.4/1.8‡		2.7	2.642
C. famata	2.4/1.8‡		2.7	2.611
Pichia guilliermondii	2.4		2.8	2.661
C. carpophila	2.1		2.5	2.532
C. fermentati	2.1		2.5	2.506
C. xestobi	2.1		2.5	2.496
P. norvegensis			1.8	1.752
C. inconspicua			2.7	2.698
Clavispora lusitaniae			3.8	
C. pararugosa			2.3	
C. rugosa			2.3	
C. catenulata			2	1.757
C. zeylanoides			4	4.055
Kluyveromyces marxianus/K. lactis			3	
C. palmioleophila/ C. pseudoglaebosa			2.8	
Saccharomyces cerevisiae			3.1	3.064

*Length estimated by the amplicon size observed on the agarose gel, with 1 kb + (Invitrogen 1 kb + λ ladder) comparison as internal standard.
†Actual length determined from the sequences.
‡Depending on the presence of the tandem repeat of the 5S rRNA gene (see Ref. 39).

in Figure 3A), PINO$^{(P)}$ (eg, CBS 1921 in Figure 3A), PINO$^{(P1)}$, and PINO$^{(P2)}$ (eg, CBS 2126 and CBS 2128, respectively, in Figure 3A). These were all distinct from the two profiles of *C. inconspicua* profile (INCO) and INCO$^{(P)}$ (Table 1, Figure 2E, and Figure 3B). For strain CBS 1922^{HF}, mislabeled as identical to the type strain CBS 1922^{T} of *C. norvegensis,* it was re-identified as *C. zeylanoides* with our PCR/RFLP method and confirmed by D1D2 sequencing (Gen Bank accession # FN554768) (Table 1 and Figures S1 and S2, at *http://jmd.amjpathol.org* for *C. zeylanoides* fingerprints).

Candida tropicalis also showed a specific profile with the IGS2 method, whereas *Clavispora lusitaniae, C. pararugosa, C. rugosa, C. catenulata, C. zeylanoides, K. marxianus (C. kefyr), K. lactis (C. sphaerica)*, *C. palmioleophila*, and *C. pseudoglaebosa* could be indentified by the whole IGSAF method (Figure 1D, Supplemental Tables S1 and S2, and Figures S1 and S2, *http://jmd.amjpathol.org*). In addition, the IGS patterns of the *Saccharomyces cerevisiae* strains and the strain used in Ultra-levure (formerly *S. boulardii*), a pro-biotic strain (Biocodex, Beauvais, France), were distinct, leading to the characterization of one clinical isolate of the Ultra-levure strain. The IGS2 sequences of the Ultra-levure and the clinical isolates were identical (Gen Bank accession numbers FN554373 and FN554374, respectively) (Table S1 and Figure S2, see *http://jmd.amjpathol.org*).[48]

Among the 270 total number of isolates tested, amplification failed for only two: *C. kefyr* CBS 834^{T} and *D. hansenii* IHEM 2325. Identification of these two strains was confirmed by NTS2 profiling[35] and by D1D2 sequencing, respectively. Among species clinically relevant *C. krusei* DNA could not be amplified using these twin IGS methods.

As the primers are nested within one another, the two PCR methods are not mutually exclusive in all species studied, and we noted that the IGS2 PCR protocol may occasionally amplify some strains also detected by the IGS PCR, such as *C. parapsilosis, C. orthopsilosis*, *C. metapsilosis, D. hansenii (C. famata), P. guilliermondii, C. fermentati, C. carpophila,* and *C. xestobii* (Table 3 for amplicon size and the schematic presentation of the IGS Figure S3, see *http://jmd.amjpathol.org*).

Discussion

Given the dramatic expansion of non-*Candida albicans* yeast infections, and the distinct antifungal susceptibility pattern of the associated species, accurate identification becomes essential for clinical management.[3,5–7,11,12,49] Using a large panel of type/reference strains and clinical isolates, we showed that a twin PCR/RFLP scheme applicable directly on yeast colonies was reliable and consistent for the closely related *Candida* species included in the most clinically relevant *Candida* species complexes. Our findings show that phenotypic method[48] leading to mis-identifications that commonly occur in the cases of *D. hansenii/C. famata* and *P. guilliermondii* (*C. guilliermondii*), or of *C. inconspicua* and *C. norvengensis,* can be easily corrected with our strategy. Owing to the decreased susceptibility of *C. guilliermondii* strains to echinocandins compounds, and the consensual recommendations for their use in the first-line therapy of candidiasis, progresses to reduce mis-identification between *C. guilliermondii* and *C. famata* may have significant therapeutic impact.[13,19,22,30] In addition, we confirmed that isolation of *C. famata*, mainly associated with dairy products, actually has a much lower clinical prevalence than believed before,[30] as opposed to *C. guillermondii*, a yeast more adapted to live in close contact with human body that emerges to be more frequently involved in clinical settings. Accurate species identification among the *C. parapsilosis* complex is also becoming crucial in clinical management.[12,50] The rapid detection of *C. parapsilosis* is now clinically relevant because the revised recommendations for the treatment of candidiasis favor fluconazole in comparison with echinocandins as first-line therapy for infections due to this species.[22] This species was detected and easily identified by our twin PCR/RFLP method given that all *C. parapsilosis* strains in this study constantly exhibited the same IGSAF profile. The IGS fingerprints showed also specific profiles for *C. guilliermondii, C. fermentati, C. carpophila,* and *C. xestobii*, whereas these species cannot be distinguished using D1D2 or ITS sequencing.[51]

Notably, we observed a specific IGS profile for the Ultra-levure *Saccharomyces cerevisiae* strain (formerly *S. boulardii*), leading to the identification of one of its clinical isolates. Beneficial effects of this pro-biotic strain remain controversial, as its ability to infect patients has been demonstrated in case of improper handling in the intensive care units.[52,53] Considering the unsolved question of the virulence of several *S. cerevisiae* strains, the differentiation we achieved with the IGS fingerprinting and/or sequencing methods may also be of importance in this field.

As similar conditions were used for the two protocols, we suggest to perform them together in a single run and then to apply the following algorithm. Three situations, in order of decreasing probability, are likely. First, if only the IGS2 method leads to an amplification of the target strain, identification of one of the most frequent clinically relevant species can be presumed according to the amplicon size (Table 3) and should be confirmed by *Nla*III fingerprints. Second, if only the IGS method leads to amplification, the *Alu*I restriction is recommended. A third, probably less likely, possibility would be that the two PCR methods yield amplicons (Table 3 for amplicon size). In this case, *Alu*I restriction should be carried out to complete identification. Amplicon sizes may be estimated either by eye or digitally on the gel after normalization. However, some studies from Chen and colleagues[54] described automated capillary electrophoresis means to distinguish species of *Candida* according to the amplicon size, but later abandoned it due to lack of uniformity

Recently, mass spectrometry using matrix assisted laser desorption/ionization-time of flight technology has been developed for microbiological identification and showed very promising results for rapid and reliable determination of the most important yeast species isolated in the clinical setting.[55–57] However, to date, mass spectrometry spectra of closely related species forming the major clinical *Candida* complexes and of unusual species have not been reported and have not been introduced into the reference databases.[56,57] Thus, additional inputs into the current databases using strains with unambiguous molecular-based delineation are required to allow accurate identification of these species for which, in particular, phenotypical methods are unsatisfactory. The panel of strains identified here by our PCR/RFLP and by sequencing may serve as a reference for the expansion of the matrix assisted laser desorption/ionization-time of flight databases.

One important limitation of our strategy is that *C. krusei* cannot be amplified by the current set of primers we use. This yeast is intrinsically resistant to azole compounds; thus, its rapid characterization is critical. With the exception of the specific condition of patients with hematologic malignancies in whom it occurs in as many as 24% of invasive candidiasis cases; this species remains rarely isolated and accounts for less than 4% of the isolates.[6–8,11,12] However, phenotypic conventional recognition of *C. krusei* is fast and reliable using presumptive identification tests, such as typical surface pellicle formation, color of the colonies on BBL CHROMagar Candida plates (BD Biosciences, Le Pont de Claix, France) with pink center and white edge, and confirmation within 15 minutes with a latex-agglutination test.[58,59] Other limitations may stem from the existence of additional polymorphisms that could have escaped our screening. In addition, it should be mentioned that this method was validated only for *Candida* species, and the degree to which other genera of pathogenic fungi may produce similar band patterns is not known.

In this report, we described the results of the identification of *Candida* reference and clinical isolates using two IGS amplification methods followed by comparison of fingerprints. These two PCR/RFLP methods targeting the IGS locus may significantly improve recognition of yeasts, in particular when phenotypic methods are unsatisfactory and lead to mis-identifications. These methods may be used as a reference molecular tool along or alternatively to D1D2 or ITS sequencing, nowadays presenting some weakness due to too much mislabeling sequences deposited in public databases.

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